Nonselective Assembly of Fibrillin 1 and Fibrillin 2 in the Rodent Ocular Zonule and in Cultured Cells: Implications for Marfan Syndrome

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Purpose. Fibrillins are the major constituent of tissue microfibrils, which form the ocular zonule. In Marfan syndrome (MFS), *FBN1* mutations lead to ectopia lentis. The goal of this work was to investigate zonule composition and formation in fibrillin-deficient and wild-type mice.

Methods. Immunofluorescence staining of eyes from wild-type, *Fbn1*-deficient, and *Fbn2*-deficient mice, as well as other species, was performed using monospecific fibrillin 1 and fibrillin 2 antibodies. The zonule of *Fbn1*-deficient and *Fbn2*-deficient mice was studied by electron microscopy. Microfibril formation in vitro was evaluated by immunofluorescence microscopy of cultured nonpigmented ciliary epithelial cells and fibroblasts.

Results. A zonule was present in both *Fbn1*-deficient and *Fbn2*-deficient mouse eyes. Immunofluorescence demonstrated that the zonule of *Fbn1*-deficient mice, wild-type mice, rats, and hamsters contained fibrillin 2. The zonule of *Fbn2*$^{-/-}$ mice contained fibrillin 1. Fibrillin 1 and fibrillin 2 colocalized in microfibrils formed in human nonpigmented ciliary epithelium cultures. Like fibrillin 1, fibrillin 2 microfibril assembly was fibronectin dependent and initiated by cell surface punctate deposits that elongated to form microfibrils.

Conclusions. These data suggest that fibrillin 1 assembly and fibrillin 2 assembly share similar mechanisms. Microfibril composition depends substantially on the local levels of fibrillin isoforms and is not highly selective in regard to the isoform. This raises the intriguing possibility that the zonule could be strengthened in MFS by inducing fibrillin 2 expression in ciliary epithelium. The presence of fibrillin 2 in the murine zonule and an intact zonule in *Fbn1*-knockout mice may limit the utility of rodent models for studying ectopia lentis in MFS.

Keywords: zonule, fibrillin, Marfan syndrome

Tissue microfibrils are primarily composed of fibrillins, which are large, secreted glycoproteins having both regulatory and structural roles.[1] The regulatory role of microfibrils reflects their binding to latent TGF-β complexes and bone morphogenetic proteins.[2,3] Their structural role is exemplified by the ciliary (or ocular) zonule, a gossamer-like cell-free structure that connects the ciliary body to the equatorial region of the lens and is composed of fibrillin microfibrils.[4] The zonule centers the ocular lens in the visual path and transmits ciliary muscle contraction forces to the lens for accommodation. The absence or weakness of or damage to the ocular zonule can result in dislocation of the lens (ectopia lentis). This anomaly is commonly present in Marfan syndrome (MFS), which results from *FBN1* mutations or, rarely, as an isolated ectopia lentis from mutations of fibrillin 1 or the microfibril-associated proteins LTBP2, ADAMTS10, ADAMTS17, and ADAMTSL4.[5–16] Fibrillin 2 deficiency causes Beals syndrome or congenital contractural arachnodactyly,[17] with skeletal manifestations resembling MFS, but ocular manifestations are rare. Mass spectrometric analysis of isolated bovine zonule identified fibrillin 1 and microfibril-associated glycoprotein 1 (MAGP-1) as the major constituents, but neither fibrillin 2 nor fibrillin 3 were found.[4] A similar analysis has not yet been done using human or mouse zonule. The mouse, which has only fibrillin 1 and fibrillin 2, has no *FBN3* functional counterpart.[18]

Previous work has shown that fibrillin 1 and fibrillin 2 can form heterotypic microfibrils.[19,20] In mice, *Fbn2* mRNA is predominantly expressed during the embryonic period, whereas *Fbn1* expression is initiated during late embryogenesis and dominates the juvenile and adult periods.[21] Recently, *Fbn2* mRNA expression was identified during early development of the ciliary body in the mouse, while *Fbn1* mRNA expression occurred later, raising the possibility that both fibrillin 1 and

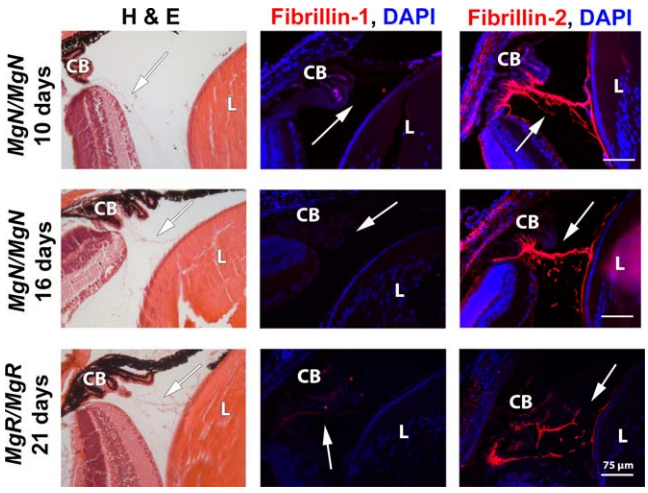

Figure 1. Fibrillin 1–null (*MgN/MgN*) and fibrillin 1–reduced (*MgR/MgR*) mice have an intact zonule composed of fibrillin 2. Staining (H&E) showed the zonule using light microscopy, and immunofluorescence microscopy was used to detect fibrillin 1 and fibrillin 2 in this anatomic structure (*red signal*). The H&E staining revealed a zonule in the 10-day-old and 16-day-old $Fbn1^{MgN/MgN}$ eyes that stained brightly with anti–fibrillin 2 antibody. The zonule bridged the gap between the ciliary processes and the equatorial surface of the lens. Lack of staining with anti–fibrillin 1 demonstrated the specificity of this antibody in immunofluorescence. The 21-day-old $Fbn1^{MgR/MgR}$ eyes also had an intact zonule evident by H&E staining. While fibrillin 1 immunofluorescence staining in this mouse was barely detectable, fibrillin 2 signal was robust. The nuclei were stained with DAPI. CB, ciliary body; L, lens. *Arrows* point to the zonule. *Scale bars*: 75 µm.

fibrillin 2 were contained in the murine zonule.[22] However, the precise composition of microfibrils could be challenging to ascertain because fibrillin 2 is reportedly masked by fibrillin 1 in adult tissues (i.e., inaccessible to antibodies).[23]

Fbn1-deficient or *Fbn1*-mutant mice have been developed to understand the functions of fibrillin 1 and mechanisms of MFS. However, the zonule of *Fbn1*-deficient or *Fbn1*-mutant mice has not been previously characterized. Herein, we report zonule characterization in mice recessive for the $Fbn1^{MgN}$ allele in which gene ablation results in complete deficiency of fibrillin 1 (the hypomorphic $Fbn1^{MgR}$ allele), in which *Fbn1* mRNA is substantially reduced compared with normal and $Fbn2^{-/-}$ mice.[24–26] These results add to the already substantial contributions that these and other mouse models have made to understanding microfibrils and MFS.[27–29]

Surprisingly, we found an intact zonule in $Fbn1^{MgN/MgN}$ mice by histology, electron microscopy, and immunofluorescence. The zonule was composed of fibrillin 2 in $Fbn1^{MgN/MgN}$ mice, fibrillin 1 in $Fbn2^{-/-}$ mice, and both fibrillin 1 and fibrillin 2 in wild-type mice, rats, and hamsters but not in the mature human and bovine eyes. Motivated by the unexpected finding of fibrillin 2 in the rodent zonule, we undertook analysis of fibrillin 2 assembly by cells and its regulation by fibronectin, which is crucial for fibrillin 1 assembly into microfibrils. Together, these in vivo and in vitro results contribute further to an improved understanding of the ocular zonule and are relevant to the recent finding of anterior segment dysgenesis in $Fbn2^{-/-}$ mice.[30]

Methods

Antibodies

The rabbit polyclonal fibrillin 1 antibody (anti-rF6H) was raised against the recombinant C-terminal half of human fibrillin 1, and its specificity was previously demonstrated by immunoblotting and ELISA against fibrillin 1 and other matrix proteins.[31] The rabbit polyclonal antibody to fibrillin 2 was raised against the glycine-rich domain of fibrillin 2 and was previously shown to be specific by immunoblotting.[32] Additional characterization of the specificity of these antibodies in immunofluorescence is described below (see Fig. 1, Fig. 2, Supplementary Fig. S1). The mouse monoclonal fibrillin 1 antibody was against bovine zonular microfibrils and reacts with human fibrillin 1 (clone 11c1.3; Millipore, Billerica, MA). Mouse monoclonal anti-fibronectin antibodies that react with human fibronectin (clone FN-15; Sigma-Aldrich Corp., St. Louis, MO) or both human and mouse fibronectins (FBN11; Millipore) were purchased. For immunofluorescence of sections, the fibrillin 1 and fibrillin 2 rabbit antisera were used at a dilution of 1:300, and the anti–fibrillin 2 antiserum was used at 1:5 dilution for immunoelectron microscopy. In cell cultures, the monoclonal fibrillin 1 antibody and polyclonal fibrillin 2 antibody were used at 1:200 and 1:300 dilutions, respectively. For fibronectin staining, FN-15 (for human cells) and FBN11 (for mouse cells) were used at 1:1000 and 1:50 dilutions, respectively. Alexa 488–conjugated and Alexa 568–conjugated secondary antibodies (Invitrogen, Thousand Oaks, CA) were used in both tissue and culture immunofluorescence studies.

Animal and Human Eye Specimens

In all experiments using gene-targeted mice, wild-type littermate eyes were used as controls. $Fbn1^{MgN/MgN}$ (*Fbn1* null) and $Fbn2^{-/-}$ mice (provided by Francesco Ramirez) lack the products of the respective genes and were produced by homologous recombination in embryonic stem cells.[24,25] Whole eyes from the $Fbn1^{MgR/MgR}$ (R indicates reduced fibrillin) mouse, wild-type littermates, Sprague-Dawley rats, and Syrian golden hamsters were provided by various laboratories. In $Fbn1^{MgR/MgR}$ mice, *Fbn1* mRNA is produced at approximately 10% of normal levels owing to insertion in intron 18 of the neomycin resistance cassette.[26] Mice were maintained in the C57BL/6J background at the Biological Resources Unit of the Cleveland Clinic under a protocol approved by the Cleveland Clinic Institutional Animal Care and Use Committee and in adherence with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. A fresh human eye from a 72-year-old individual was obtained within 9 hours of death and provided by the Midwest Eye-Banks (Ann Arbor, MI) on ice within 48 hours of death in adherence with the Declaration of Helsinki. A fresh adult bovine eye (precise age unknown) was purchased from a local abattoir.

Immunofluorescence

Mouse eyes were fixed intact, while rat and hamster eyes were punctured to facilitate fixation. The human and bovine globes were first cut coronally, and the anterior half was quartered. These eyes were fixed overnight in 4% paraformaldehyde in PBS before paraffin embedding. Paraffin sections (6–8 µm thick) on SuperFrost Plus slides (Thermo Fisher Scientific, Inc., Waltham, MA) were baked for 3 to 4 hours at 50°C, dewaxed with Histo-Clear (National Diagnostics, Atlanta, GA), and rehydrated through graded ethanols. Antigen retrieval was performed by heating the slides in citrate-EDTA buffer (10 mM citric acid, 2 mM EDTA, 0.05% Tween-20, pH 6.2) in a domestic microwave oven three times for 1.5 minutes each at 50% power. Slides were washed two times for 2 minutes each in PBST (PBS containing 0.1% Tween-20) and for 5 minutes in PBS. Slides were incubated with blocking solution (10% normal goat serum [NGS] in PBS) at room temperature for 1 hour and

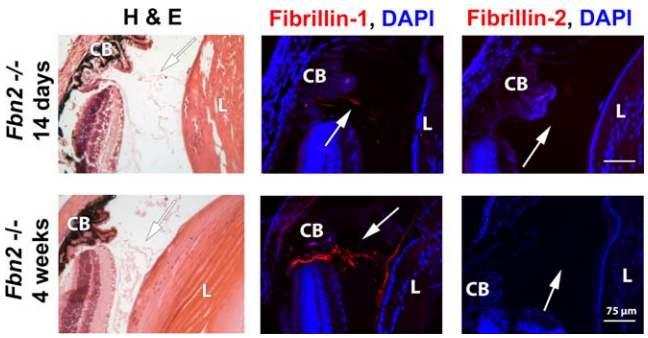

FIGURE 2. Fibrillin 2–deficient mice have an intact zonule composed of fibrillin 1. Staining (H&E) showed zonule fibers by light microscopy, and immunofluorescence microscopy was used to detect fibrillin 1 and fibrillin 2 (*red signal*). An intact zonule was observed in the *Fbn2*$^{-/-}$ mouse eye at age 14 days and 4 weeks by H&E staining. Immunofluorescence detected positive signal with the anti–fibrillin 1 antibody in the zonule. Lack of anti–fibrillin 2 staining in the *Fbn2*$^{-/-}$ mouse tissue demonstrated the specificity of this antibody. Nuclei were stained with DAPI. CB, ciliary body; L, lens. *Arrows* point to the zonule. *Scale bars*: 75 µm.

then with anti-fibrillin antibodies diluted in blocking solution overnight at 4°C. Sections were washed with PBST three times for 5 minutes each and incubated for 1 hour at room temperature with secondary antibody in blocking solution. Sections were washed three times for 10 minutes each in PBST and mounted in ProLong Gold Anti-Fading Reagent with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen), viewed using a Leica DM5500B upright microscope (Leica Microsystems, GmbH, Wetzlar, Germany), photographed with a Retiga SRV Cooled charge-coupled device camera with Liquid Crystal tunable red-green-blue (RGB) filter (QImaging, Surrey, BC, Canada), and processed using ImagePro Plus software (Media Cybernetics, Bethesda, MD).

Scanning and Transmission Electron Microscopy

For scanning electron microscopy (SEM), mouse eye globes were fixed overnight in 1.5% glutaraldehyde–1.5% paraformaldehyde in Dulbecco's modified Eagle's medium (DMEM) with a final concentration of 0.05% tannic acid at 4°C, rinsed in DMEM, and then exposed to 1% osmium tetroxide in DMEM for several hours at 4°C. The eye globes were rinsed in DMEM, dehydrated in a graded ethanol series to 100% ethanol, and dried in an Autosamdri 814 critical point dryer (Tousimis, Rockville, MD). The globes were then cut with a razor blade to expose ciliary bodies and zonula. The samples were sputter coated with gold-palladium using a Balzers 010 coater (Oerlikon Balzers Coating USA, Inc., Schaumburg, IL) and then examined at 10 kV using the high-resolution stage of an ISI DS130 scanning electron microscope (International Scientific Instruments, Milpitas, CA). For immunoelectron microscopy, small cuts were made in the posterior portion of the globe using iris scissors, and then the globes were immersed in fibrillin 2 primary antibody diluted 1:5 in DMEM overnight at 4°C. Following an extensive rinse in DMEM, the globes were immersed overnight at 4°C in 6-nm goat anti-rabbit secondary gold conjugate (Electron Microscopy Sciences, Hatfield, PA). The intact globes were rinsed extensively in DMEM, fixed in 1.5% glutaraldehyde–1.5% paraformaldehyde in DMEM with 0.05% tannic acid, rinsed, and then exposed to 1% osmium tetroxide for several hours. The samples were rinsed in DMEM, dehydrated in a graded ethanol series to 100%, washed in propylene oxide, and then infiltrated and embedded in Spurr's epoxy (Polysciences, Inc., Warrington, PA). Eighty-nanometer ultrathin sections were collected onto 1 × 2-mm single-hole Formvar-coated slot grids (Ted Pella, Inc., Redding, CA) and examined using transmission electron microscopy (TEM; G2 Tecnai; FEI, Hillsboro, OR) operated at 120 kV.

Cell Culture and Immunocytochemistry

To prepare mouse embryo fibroblasts (MEFs), embryos were dissected under sterile conditions at 14.5 days' gestation, viscera and heads were discarded, and the torso was minced with sterile scissors and digested using 0.05% trypsin and 0.53 M ethylenediamine–tetraacetic acid (Sigma-Aldrich Corp.). Enzymatically released cells were plated in monolayer culture in DMEM, 10% fetal bovine serum (FBS), 100 µ/mL penicillin, 100 µg/mL streptomycin, and MycoZap (Lonza, Basel, Switzerland). For preparation of embryonic mouse skin fibroblasts (MSFs), skin was dissected under sterile conditions, fat was excised, and defatted skin was cut into 3-mm^2 pieces, which were grown as explants in the same medium as above to obtain fibroblast outgrowth. Human dermal fibroblasts (5 × 10^4; Invitrogen), fetal bovine nuchal ligament fibroblasts (fBNLs), MEFs, or MSFs (all used before passage 10) were seeded on 8-well chamber slides and cultured in DMEM and 10% FBS with penicillin-streptomycin as stated above. Human nonpigmented ciliary epithelial cells (HNPCECs; ScienCell Research Laboratories, Carlsbad, CA) were cultured in epithelial cell medium (EpiCM; ScienCell Research Laboratories) supplemented with 2% FBS, 1% epithelial cell growth supplement (EPiCGS; ScienCell Research Laboratories), and 1% penicillin-streptomycin solution (P/S; ScienCell Research Laboratories). Their epithelial origin was confirmed by strong cytokeratin expression (data not shown). The cells were cultured for up to 7 days, with a change of medium every 48 hours. For immunofluorescence, cells were fixed for 7 minutes with ice-cold methanol, blocked with 5% NGS in PBS, and incubated with monoclonal anti–fibrillin 1, polyclonal anti–fibrillin 2, and/or monoclonal anti-fibronectin antibodies at 4°C overnight. Cells were washed three times in PBST and incubated with secondary antibody for 1 hour at room temperature, washed three times in PBST, and washed once with PBS. Nuclei were stained with ProLong Gold Anti-Fading Reagent with DAPI (Invitrogen). In order to determine whether fibronectin fibrillogenesis affected fibrillin 2 assembly, FUD (a 56-residue recombinant polypeptide based on the functional upstream domain of *Streptococcus pyogenes* protein F1)[33] at concentrations of 0, 50, 75, or 100 nmol/L was added to cells for the duration of culture, with replenishment at each medium change, followed by fibrillin 2 immunofluorescence after 5 days. FUD interacts with secreted fibronectin to prevent assembly of fibronectin fibrils.[33] Cultures of *Fn*-deficient fibroblasts[34] utilized FBS from which fibronectin was depleted.

RESULTS

Fibrillin 1–Deficient Mice Have an Intact Ciliary Zonule Comprising Fibrillin 2

The zonule was present in 10-day-old and 16-day-old *Fbn1*$^{MgN/MgN}$ mice (Fig. 1, hematoxylin-eosin [H&E] stain). Arising from the ciliary processes bordering the pars plana, the zonule fibers extended to the equatorial region of the lens. The *Fbn1*$^{MgN/MgN}$ mouse zonule did not stain with the polyclonal anti–fibrillin 1 antibody, nor was there staining elsewhere in the eyes in *Fbn1*$^{MgN/MgN}$ mice (Fig. 1), in contrast to staining of wild-type or fibrillin 2–deficient mice, demonstrating antibody specificity (Figs. 2, 3). Staining of cultured *Fbn1*$^{MgN/MgN}$ cells provided additional evidence for specificity (Supplementary Fig. S1).

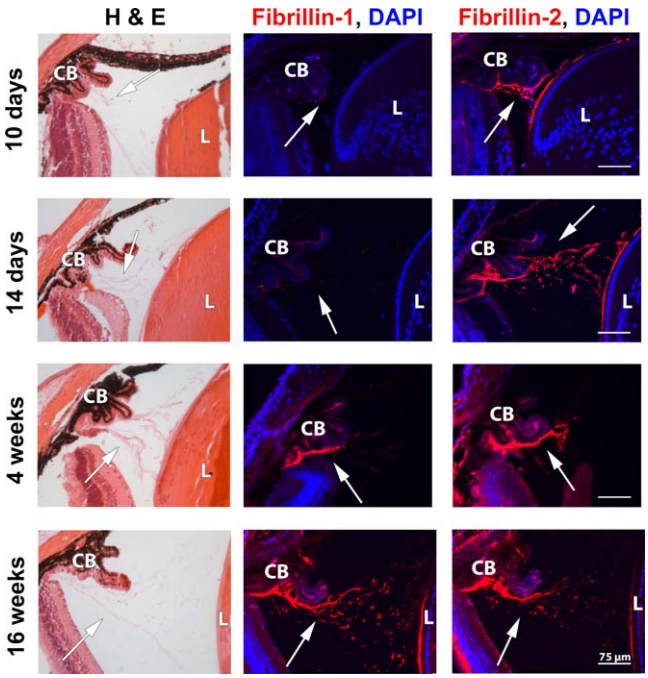

FIGURE 3. Fibrillin 2 is the predominant component of the zonule of juvenile wild-type mice. Staining (H&E) showed zonule fibers by light microscopy in the 10-day-old, 14-day-old, 4-week-old, and 16-week-old wild-type mouse eyes. Immunofluorescence microscopy using monospecific antibodies detected both fibrillin 1 and fibrillin 2 (*red signal*) in the wild-type zonule. Anti–fibrillin 1 antibody stained the wild-type zonule faintly in juvenile eyes (10 days and 14 days) but more robustly in the mature eyes (4 weeks and 16 weeks). Fibrillin 2 predominates at age 10 days and 14 days and persists in older mice. Nuclei were stained with DAPI. CB, ciliary body; L, lens. *Arrows* point to the zonule. *Scale bars*: 75 μm.

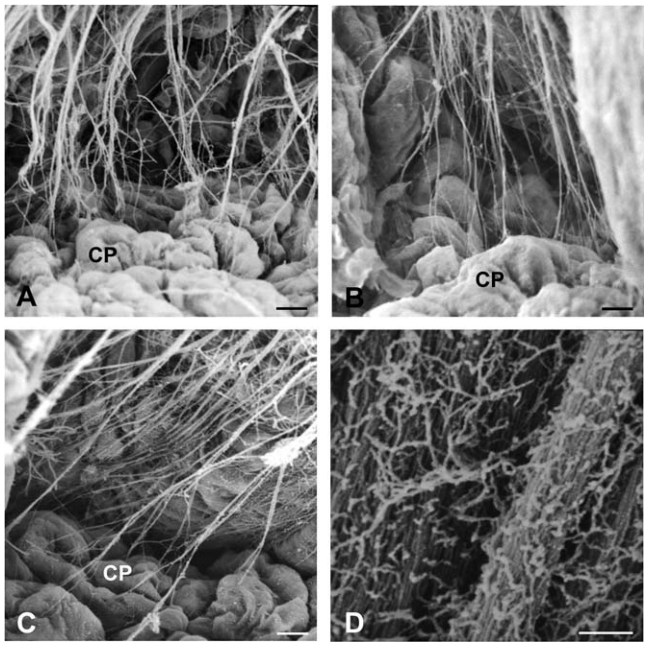

FIGURE 4. Scanning electron microscopy demonstrates the presence of the zonule in 18-day-old wild-type (**A**), 20-day-old *Fbn2*$^{-/-}$ (**B**), and 18-day-old *Fbn1*$^{MgN/MgN}$ (**C**) eye globes. (**A–C**) The structures of the zonula are similar in **A** through **C**, although there appear to be fewer and slimmer microfibril bundles in *Fbn2*$^{-/-}$ (**B**) and *Fbn1*$^{MgN/MgN}$ (**C**) mice. (**D**) At higher magnification, structures of the size and character expected of fibrillin microfibrils, with loose bridging fibers, are seen to compose the zonular fibers in the *Fbn1*$^{MgN/MgN}$ eye globe (**D**). CP, ciliary processes. *Scale bars*: 2 μm (**A–C**) and 0.5 μm (**D**).

Staining with anti–fibrillin 2 antibody identified fibrillin 2 in the entire zonular apparatus of 10-day-old and 16-day-old *Fbn1*$^{MgN/MgN}$ mice (Fig. 1), including zonule fibers extending from the ciliary body to the lens, the inner limiting membrane of the ciliary processes, and the equatorial surface of the lens capsule. Because *Fbn1*$^{MgN/MgN}$ mice die between age 2 to 3 weeks, we could not determine the fate of their zonule beyond this period.[25] In 21-day-old *Fbn1*$^{MgR/MgR}$ mice, the zonule was also histologically evident; unlike the *Fbn1*$^{MgN/MgN}$ zonule, weak fibrillin 1 immunostaining was indeed detectable in the *Fbn1*$^{MgR/MgR}$ zonule, consistent with reduced *Fbn1* expression in these mice. As in the *Fbn1*$^{MgN/MgN}$ zonule, strong fibrillin 2 staining was seen in the *Fbn1*$^{MgR/MgR}$ zonule (Fig. 1).

Fbn2$^{-/-}$ Eyes Have an Intact Zonule Composed of Fibrillin 1

The 14-day-old and 4-week-old *Fbn2*$^{-/-}$ mice had a histologically evident zonule (Fig. 2) that stained with the fibrillin 1 antibody but not with the fibrillin 2 antibody, as expected. In 14-day-old *Fbn2*$^{-/-}$ mice, fibrillin 1 staining was most evident in the zonule closest to the ciliary body, whereas at age 4 weeks fibrillin 1 staining spanned the zonule and was also seen on the equatorial surface of the lens (Fig. 2).

Fibrillin 2 Is a Major Component of the Ciliary Zonule in Mice

We asked whether the presence of fibrillin 2 in the *Fbn1*$^{MgN/MgN}$ zonule was a compensatory response to fibrillin 1 deficiency or whether fibrillin 2 was a component of the wild-type mouse zonule. Fibrillin 1 and fibrillin 2 immunofluorescence indicated that both fibrillins were components of the wild-type mouse zonule at age 10 days, 14 days, 4 weeks, and 16 weeks (Fig. 3). Bright fibrillin 2 staining was seen in the juvenile zonule (10 days and 14 days), although fibrillin 1 staining was much weaker in this period. However, by age 4 weeks and 16 weeks, fibrillin 1 staining was stronger, with persistence of fibrillin 2 staining at 4 weeks and 16 weeks (Fig. 3). Although the staining with these two antibodies cannot be directly compared, this result suggests that fibrillin 2 precedes fibrillin 1 in the wild-type zonule and persists as a prominent component through to maturity.

Ultrastructure of the Zonule in *Fbn1*-Knockout and *Fbn2*-Knockout Mice

Scanning electron microscopy of 18-day-old wild-type zonule demonstrated typical microfibril bundles arising as fine fibers from the ciliary processes (Fig. 4A). Both 20-day-old *Fbn2*$^{-/-}$ zonule (Fig. 4B) and 18-day-old *Fbn1*$^{MgN/MgN}$ zonule (Fig. 4C) had comparable structures. At higher magnification, these zonula comprised tightly bundled aggregates of microfibrils with loosely arranged bridging fibers (Fig. 4D). The overall appearance of the *Fbn1*-mutant and *Fbn2*-mutant zonula in SEM suggested a slight paucity of fibers relative to wild-type zonule; although there was much variation in image size, the images also conveyed the qualitative overall impression of slightly thinner fibers in the two mutant zonula (Figs. 4A–C). In TEM, low-magnification images showed zonule fibers arising from the valley between adjacent ciliary processes and spanning the vitreous to the lens (Fig. 5A). Microfibrils incubated with anti–fibrillin 1 antibody were previously

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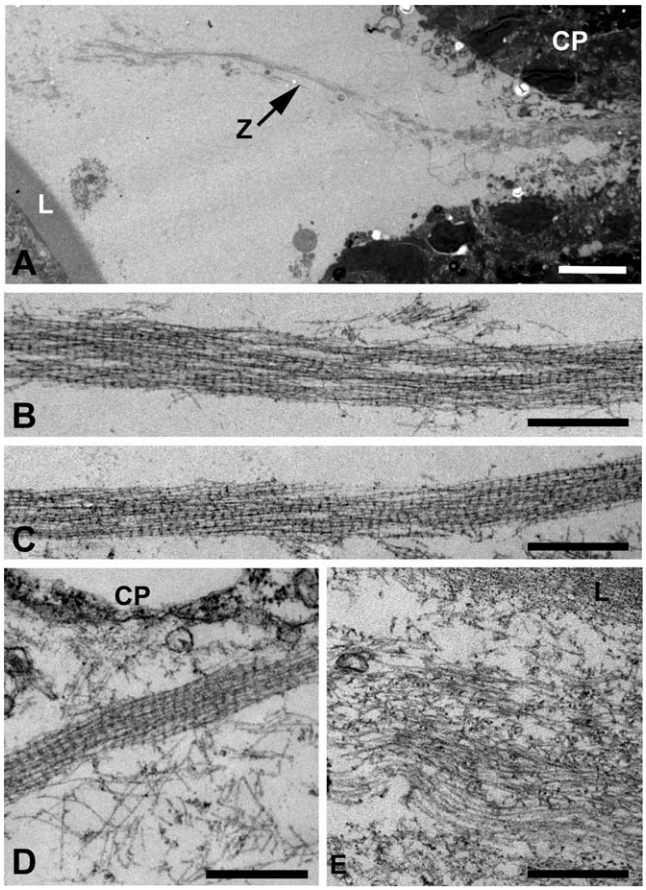

FIGURE 5. Transmission electron microscopy demonstrates comparable microfibril structure in the *Fbn1*$^{MgN/MgN}$ (**A**, **C**, **D**), wild-type (**B**), and *Fbn2*$^{-/-}$ (**E**) zonula. (**A**) Low-magnification TEM demonstrates a longitudinally sectioned zonule (Z) in a 7-day-old *Fbn1*$^{MgN/MgN}$ eye globe arising from the ciliary process (CP) and nearly intersecting the capsule of the lens (L). (**B**) Microfibrils within the zonula in the wild-type eye are aligned laterally in bundles and show a banding periodicity of approximately 50 nm when exposed to fibrillin 2 antibody. (**C**) Lateral alignment of microfibrils and periodicity matching the wild-type zonule are also demonstrated in the *Fbn1*$^{MgN/MgN}$ zonule after labeling with fibrillin 2 antibody. (**D**) The midzones of zonula are represented in (**B**) and (**C**) above; this portion of the *Fbn1*$^{MgN/MgN}$ zonule labeled with anti–fibrillin 2 intersects the basement membrane of a ciliary process. (**E**) Microfibrils adjacent to the lens (L) in the *Fbn2*$^{-/-}$ eye demonstrate no periodicity after exposure to fibrillin 2 antibody. *Scale bars*: 10 μm (**A**) and 0.5 μm (**B**–**E**).

demonstrated to manifest a periodicity due to binding to the antibody.[35] We utilized this phenomenon for identification of fibrillin 2–containing microfibrils because the gold-labeled secondary antibody did not penetrate efficiently. In wild-type and *Fbn1*$^{MgN/MgN}$ eyes incubated with the anti–fibrillin 2 antibody, an antibody-dependent periodicity was seen in the zonule fibers (Figs. 5B–D). The images in Figures 5B and 5C represent the middle region of the zonule, with a similar appearance found in the zonule close to the ciliary processes (Fig. 5D). The *Fbn2*$^{-/-}$ zonule was stained as a control and did not demonstrate antibody-dependent periodicity with anti–fibrillin 2 antibody (Fig. 5E). Taken together, the SEM and TEM studies demonstrated that a zonule that is ultrastructurally comparable to that of the wild-type mouse eye developed in the absence of either fibrillin 1 or fibrillin 2.

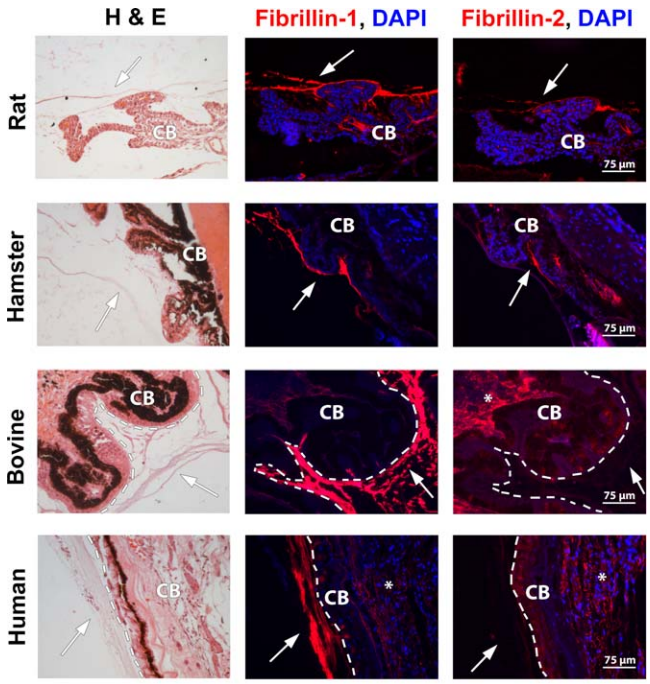

FIGURE 6. Fibrillin 2 is a component of the hamster and rat zonula but is absent from human and bovine zonula. Staining (H&E) showed zonule fibers by light microscopy in rat, hamster, bovine, and human eyes. By immunofluorescence using a monospecific antibody, fibrillin 1 was detected in the zonula of all four species (*red signal*). Staining with anti–fibrillin 2 demonstrated that fibrillin 2 is a component of the rat and hamster zonula, while it is absent in bovine and human zonula, despite specifically staining microfibrils elsewhere in eyes of these species (*asterisks* show fibrillin 1 and fibrillin 2 staining in the interior of ciliary processes). The *broken white line* outlines the inner limiting membrane of the ciliary nonpigmented epithelium. Nuclei were stained with DAPI. CB, ciliary body; L, lens. *Arrows* point to the zonule. *Scale bars*: 75 μm.

Fibrillin 2 Is a Constitutive Zonule Component in Small Rodents

Fibrillin 1 and fibrillin 2 were present in the zonule of the mature rat and hamster, as well as that of the mature mouse (Fig. 6). As in the mouse eye, in rat and hamster fibrillin 1 and fibrillin 2 were observed along the inner limiting membrane of the ciliary body (Fig. 6) and the equatorial surface of the lens (data not shown). In contrast, for the bovine or human zonule no staining was obtained with fibrillin 2 antibody (Fig. 6); however, microfibrils in the interior stroma of the ciliary processes and elsewhere in the eyes of these species were clearly stained with this antibody, indicating that the absence of fibrillin 2 in the zonule was not a consequence of nonreactivity to fibrillin 2 of the respective species (Fig. 6).

Fibrillin 1 and Fibrillin 2 Colocalize in Microfibrils Formed by Cultured Cells

The anti–fibrillin 1 polyclonal antibody stained microfibrils in wild-type MSF cultures but not in *Fbn1*$^{MgN/MgN}$ cultures. In *Fbn2*$^{-/-}$ MSF cultures, microfibrils stained with anti–fibrillin 1 antibody but not with anti–fibrillin 2, indicating specificity of these antibodies (Supplementary Fig. S1). Assembly of microfibrils by cells from mice lacking each fibrillin suggests that fibrillin 1 microfibril assembly does not depend on fibrillin 2 and vice versa. In HNPCECs and MG63 cell cultures stained for

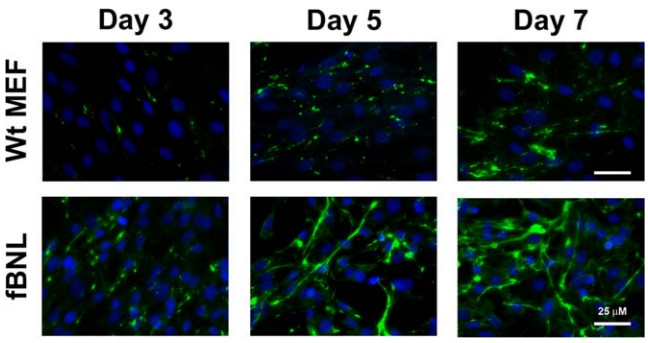

FIGURE 7. Punctate assemblies of fibrillin 2 precede the appearance of microfibrils in the extracellular matrix. Monolayer cultures of wild-type (Wt) MEFs and fBNLs are shown. In both cell types, fibrillin 2 immunofluorescence (*green signal*) first appeared as punctate or short linear cell–associated structures with extensive microfibrils visible after 5 days and 7 days (*green signal*). Nuclei were stained with DAPI. *Scale bars*: 25 μm.

both fibrillin 1 and fibrillin 2 (Supplementary Fig. S2) and in wild-type MEFs (data not shown), there was complete overlap of staining with the respective antibodies. This colocalization supports the formation of heterotypic fibrils, with the caveat that at the level of resolution used herein colocalization could also be explained by homotypic but coaligned fibrillin 1 and fibrillin 2 assemblies. To evaluate the process of fibrillin 2 incorporation into microfibrils, we analyzed microfibril formation in wild-type and fBNL cells. In wild-type MEFs, staining with the fibrillin 2 antibody showed short cell surface punctate structures 2 to 3 days after initiating cultures (Fig. 7). These punctate deposits were succeeded by short linear structures at day 5 and by extensive microfibril networks at day 7 (Fig. 7). A qualitatively and temporally similar process was observed in fBNL cells (Fig. 7). Although *Fbn2* mRNA is primarily expressed in the embryonic period in vivo, fibrillin 2–containing microfibrils were seen not only in MEFs and fBNLs but also in the MG63 cell line and HNPCECs, which are not of embryonic origin (Supplementary Fig. S2).

Fibrillin 2 Microfibril Assembly Requires Fibronectin

Inhibition of fibronectin assembly in HNPCECs (Fig. 8A) and MEFs (Supplementary Fig. S3) using FUD led to reduced fibrillin 2 microfibril assembly. $Fn^{-/-}$ fibroblasts did not form fibrillin 1 or fibrillin 2 microfibrils; $Fn^{+/-}$ fibroblasts formed fibrillin 1–containing microfibrils but not fibrillin 2–containing microfibrils under similar culture conditions (Fig. 8B). Fibrillin 2 colocalized with fibronectin in microfibrils formed by wild-type and *Fbn1*-deficient MEFs (Fig. 8C, Supplementary Fig. S3). In a manner similar to that of fibrillin 1, these findings demonstrated that fibrillin 2 microfibril assembly depends on fibronectin network formation in the extracellular matrix.[36,37]

DISCUSSION

In contrast to extensive analysis of the cardiovascular, pulmonary, and musculoskeletal systems of *Fbn1*-deficient or *Fbn1*-mutant mice in the context of MFS,[26,29,32,38] their ocular zonule has not been characterized previously. The present study was undertaken not only because ectopia lentis is a major clinical feature of MFS but also because of recently discovered mutations in fibrillin-binding proteins that cause ectopia lentis.[8–16,22,29,32,38–40] Previously, Mir et al.[41] and Traboulsi and colleagues[42] demonstrated that the zonule fibers

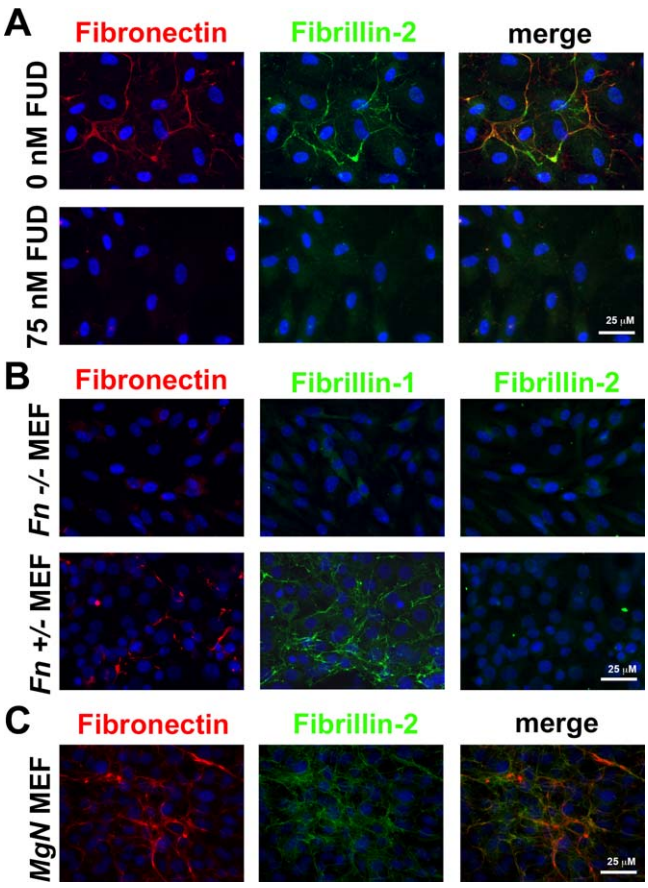

FIGURE 8. Fibrillin 2 microfibril assembly requires fibronectin. (**A**) In cultures of HNPCECs, fibrillin 2 immunofluorescence (*green*) localized with fibronectin immunofluorescence (*red*) as shown in the merged panel. Upon inhibition of fibronectin assembly with 75 nmol/L FUD, fibronectin assembly was prevented, with concomitant disappearance of fibrillin 2. (**B**) $Fn^{-/-}$ fibroblasts and $Fn^{+/-}$ fibroblasts were cultured using fibronectin-depleted serum. $Fn^{-/-}$ fibroblasts assembled neither fibrillin 1 nor fibrillin 2 into microfibrils. In the $Fn^{+/-}$ fibroblast cultures, fibronectin microfibrils were formed, there were abundant fibrillin 1 microfibrils, but no fibrillin 2 was detected. (**C**) Fibronectin and fibrillin 2 immunofluorescence colocalized in $Fbn1^{MgN/MgN}$ MEFs. *Scale bars*: 25 μm or 50 μm, as indicated.

appeared fragmented and irregular in the MFS eye. In cattle with MFS, the affected zonula were fragile, wavy, and loosely arranged.[43] In contrast to MFS in humans and cows, in which one normal *FBN1* allele is present, we thus expected that the zonule of $Fbn1^{MgN/MgN}$ mice would be completely absent because these mice make no fibrillin 1 whatsoever.

The present work provided several novel findings. These include the following: (1) unequivocal evidence for an architecturally intact zonule composed of fibrillin 2 in the fibrillin 1–deficient eyes using histology, SEM, and TEM; (2) evidence that the zonula of mouse, rat, and hamster contain both fibrillin 1 and fibrillin 2; (3) analysis of fibrillin 2 and fibrillin 1 distribution in the normal and fibrillin 1–deficient or fibrillin 2–deficient mouse zonule, respectively; and (4) insights on the formation of fibrillin 2 microfibrils by cultured cells and demonstration of the crucial role of fibronectin in fibrillin 2 microfibril assembly.

Based on previous knowledge that fibrillin 1 but not fibrillin 2 mutations led to ectopia lentis in humans, as well as previous investigations of the bovine (*Bos taurus*), Japanese monkey (*Macaca fuscata*), and human zonule using biochemical and immunochemical methods, it was believed until recently that

only fibrillin 1 had a significant role in zonule microfibrils.[4–6,30,41,44,45] In the absence of a comprehensive analysis of embryonic human eyes, however, it is presently unclear whether fibrillin 2 is a component of the human zonule during its development.

Our findings support and complement a recent comprehensive analysis of fibrillin mRNA expression in the mouse eye using in situ hybridization and immunofluorescence.[22] This study demonstrated that expression of *Fbn2* in the ciliary body dominated the embryonic and neonatal periods, with a dramatic decline postnatally as *Fbn1* mRNA expression increased. Taken together with our findings in fibrillin 1–deficient and $Fbn2^{-/-}$ mouse eyes, we conclude that, despite the clear temporal sequence of *Fbn2* and *Fbn1* mRNA expression, mouse zonule formation may be influenced by, but does not absolutely depend on, the presence of either fibrillin.

Previous work showed that heterotypic fibrillin microfibrils prevailed in tissues and that cells producing both fibrillin 1 and fibrillin 2 assemble heterotypic fibrils.[20] This demonstrated that heterotypic polymerization occurred readily, although perhaps not obligatorily. To extend these observations in vitro, we used HNPCECs, which are derived from the nonpigmented epithelium of the ciliary body that produces the zonule, and MSFs.[46] In the extracellular matrix produced by these cells, fibrillin 1 and fibrillin 2–costained microfibrils predominated, without apparent bias for inclusion of either fibrillin 1 or fibrillin 2. This finding suggests that whichever fibrillin is available is incorporated during microfibril assembly by these cells.

Fibronectin fibrillogenesis occurs early in most cells in cultures and is a prerequisite for formation of fibrillin 1–containing microfibrils.[37] The experiments presented herein using fibronectin-deficient fibroblasts or using HNPCECs in which fibronectin assembly was impaired indicated that like fibrillin 1 assembly[36] fibrillin 2 assembly was also dependent on fibronectin fibrillogenesis. Despite the similarities between fibrillin 1 and fibrillin 2 assembly observed herein, the present study also revealed that fibrillin 2 assembly was more severely impaired than fibrillin 1 assembly in $Fn^{+/-}$ cells, for reasons that are presently unclear.

Recently, Charbonneau et al.[23] raised the possibility that fibrillin 2 immunoreactivity can be masked by fibrillin 1, suggesting that fibrillin 2 microfibrils may persist in adult tissues but be less detectable by antibodies than fibrillin 1**.** Our observations did not suggest masking of the epitope recognized by the fibrillin 2 antibody used herein. Indeed, in 16-week-old wild-type mice, fibrillin 2 was still accessible to the antibody in zonule microfibrils and was therefore not masked by fibrillin 1.

In conclusion, the present analysis presents novel insights on the differences in zonule composition across species, as well as the process of microfibril assembly in vitro. The findings suggest that rodents may not be the best animal model for investigating ectopia lentis in MFS. However, the new finding that the rodent zonule is composed of both fibrillin 1 and fibrillin 2, as well as that despite the absence of fibrillin 1 a zonule forms from fibrillin 2 microfibrils, could encourage a novel perspective on potential treatment options for ectopia lentis in MFS. Currently, there is no way of strengthening the zonule in the MFS eye. The experimental observations raise the interesting possibility that induced expression of fibrillin 2 (or speculatively fibrillin 3) in the MFS nonpigmented ciliary epithelium during the period of zonule formation could compensate for fibrillin 1 deficiency in MFS or isolated ectopia lentis. However, the long-term fate of the fibrillin 2 zonule in $Fbn1^{MgN/MgN}$ mice is unclear because these mice die between age 2 and 3 weeks.[25] It is possible that a zonule comprising solely fibrillin 2 may have altered functionality or stability relative to the zonule of wild-type mice.

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